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Co-activation of GABA receptors inhibits the JNK3 apoptotic pathway via the disassembly of the GluR6-PSD95-MLK3 signaling module in cerebral ischemic-reperfusion

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Abstract In this study, we investigated whether the increase of inhibitory γ-amino butyric acid (GABA) signal suppresses the excitatory glutamate signal induced by cerebral ischemia and the underlying mechanisms. In global cerebral ischemia, focal cerebral ischemia and oxygen-glucose deprivation, application of muscimol and baclofen, agonists of GABA(A) receptor and GABA(B) receptor, exerted neuroprotection. The agonists inhibited the increased assembly of the GluR6-PSD-95-MLK3 module induced by cerebral ischemia and the activation of the MLK3-MKK4/7-JNK3 cascade. Our results suggest that stimulation of the inhibitory GABA receptors can attenuate the excitatory JNK3 apoptotic signaling pathway via inhibiting the increased assembly of the GluR6-PSD-95-MLK3 signaling module in cerebral ischemia.

Keywords: Cerebral ischemia; Muscimol; Baclofen; JNK pathway; Neuroprotection

1. Introduction

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Thus GABA release in cerebral ischemia is also likely to be important, because it can alter concentration of extracellular glutamate [1]. GABA activates three types of specific receptors: GABA(A), GABA(B) and GABA(C) receptors. At present, little is known about GABA(C) receptor-mediated events. GABA(B) receptors are metabotropic and seven transmembrane receptors that are G-proteins-coupled and mediate the GABA signaling via several second messenger pathways and Ca^{2+} and K^+ ion channels, whilst stimulation of ionotropic GABA(A) receptors results in opening the chloride channel [2]. Previous studies suggest that the pharmacologically increased level of GABA activity is correlated with lessened degree of brain ischemic injury [3–5]. Other investigators, however, have not been able to demonstrate the benefit about those activating GABA receptors in ischemia, particularly those agents relatively specific for the GABA-B receptor [6]. Therefore, the possible neuroprotective mechanism of GABA receptors activation in cerebral ischemia remains to be elucidated.

Our recent studies show that GluR6 (subunit of KA receptor) are activated upon cerebral ischemia, and the assembling of GluR6-PSD-95-MLK3 module, MLK3 autophosphorylation and the downstream phosphorylation of MKK4/7, JNK3, c-Jun and Bcl-2 increased markedly in ischemia/reperfusion. Also, the expression of Fas-Ligand, the release of Bax from Bcl-2/Bax dimmers and the release of cytochrome *c* from mitochondria markedly increased. Consequently, the activation of Caspase 3 lead to delayed neuronal death in the hippocampal CA1 subfield [7–9]. Thus, the present study was aimed to identify whether stimulation of the inhibitory GABA receptors have neuroprotective effects thorough inhibiting the excitatory JNK3 apoptotic pathway via disassembly of GluR6-PSD95-MLK3 signaling module in cerebral ischemic-reperfusion, so as to find better therapeutic approach to cerebral ischemia.

2. Materials and methods

2.1. Animal model of ischemia

Adult male SD rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 250–300 g were used. Four-vessel occlusion (4-VO) cerebral ischemia was induced as described before [1]. Briefly, under anaesthesia with chloral hydrate (350 mg/kg, i.p.), vertebral arteries were electrocauterized and common carotid arteries were exposed. Rats were allowed to recover for 24 h and fasted overnight. Ischemia was induced by occluding the common arteries with aneurysm clips. Rats which lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light during ischemia were selected for the experiments. An EEG was monitored to ensure isoelectricity within 30 s after carotid artery occlusion. Carotid artery blood flow was restored by releasing the clips. Rectal temperature was controlled at 36.5–37.5 °C before and after ischemia–reperfusion and after treatment with drugs via a temperature-regulated heating pad. Sham control animals received the same surgical procedures except that carotid arteries were not occluded.

Focal cerebral ischemia was induced by the intraluminal suture MCAo method as described in Longa et al. [2]. Briefly, the left common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were exposed through a midline incision of the

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Abbreviations: NMDA, *N*-methyl-D-aspartic acid; GABA, γ-amino butyric acid

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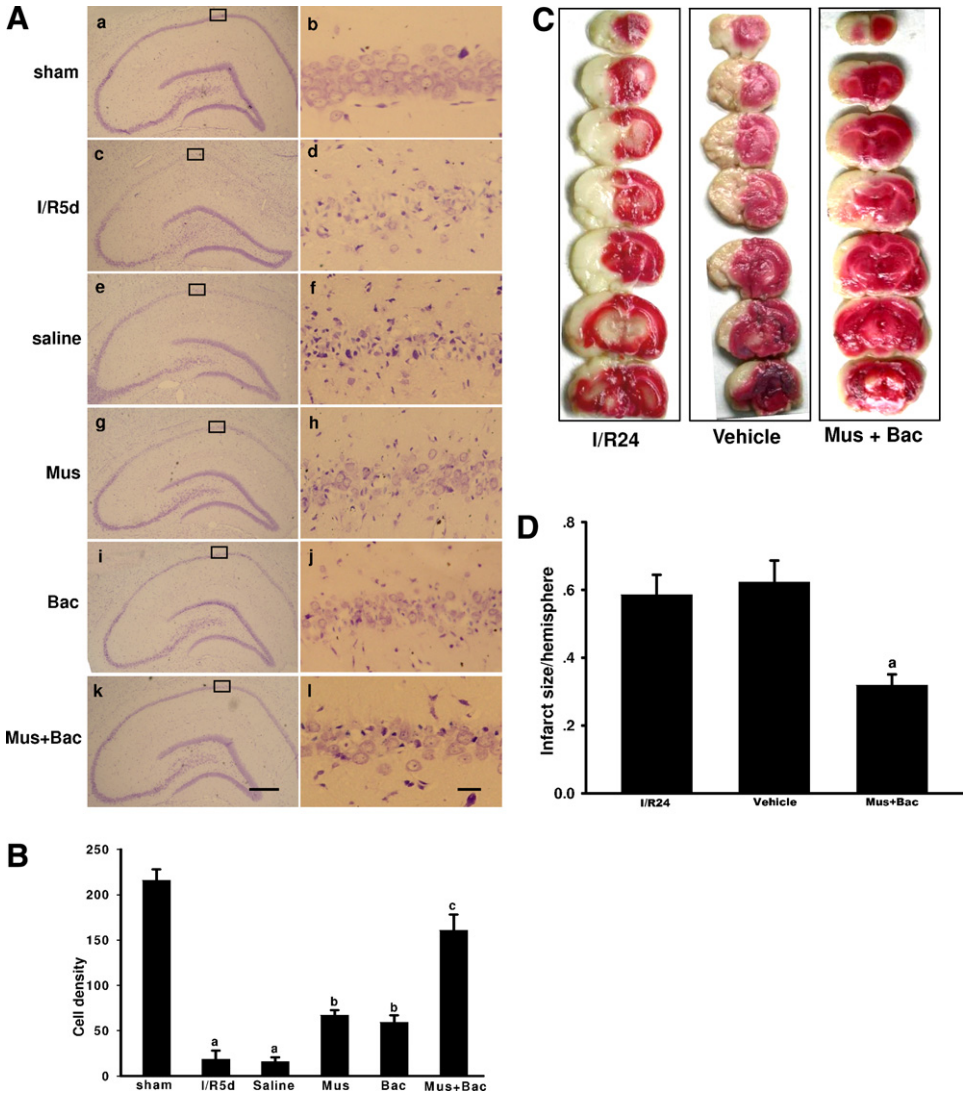

Fig. 1. Effects of muscimol and/or baclofen on neuronal injury in cerebral ischemia and on infarction size in MCAO model. (A) Cresyl violet staining was performed on the sections from the hippocampi in sham (a, b), and rats subjected to 5 days of reperfusion after 15 min of ischemia (c, d), administration of the vehicle (e, f), muscimol (1 mg/kg) (g, h), baclofen (20 mg/kg) (i, j), muscimol and baclofen (k, l) before ischemia. Treatment with muscimol and/or baclofen significantly increased the number of the survival neurons at 5 days of reperfusion in the CA1 region. (B) Quantitative analysis of the protective effects of muscimol and/or baclofen against ischemic injury. Data were obtained from five independent animals and a typical experiment is presented. Cell density was expressed as the number of cells per 1-mm length of the CA1 pyramidal cells counted under a light microscope. Scale bars: $k = 400$ μm; $l = 10$ μm. $^{a}P < 0.05$ versus sham control, $^{b}P < 0.05$ versus saline treatment group. $^{c}P < 0.05$ versus muscimol or baclofen treatment group. (C) Muscimol and baclofen reduced the infarction volume in 24 h of MCAO rats. The MCA was occluded by a thread inserted through the carotid artery. The animal was sacrificed after 24 h, and brain tissue was cut and stained with TTC. The non-stained (white) area indicates infarct size. (D) The amount of injury was expressed as a ratio of the total infarct volume to the total volume of the damaged hemisphere. $^{a}P < 0.05$ versus saline treatment group ($n = 5$ per group).

neck. A 3-0 silica gel-coated nylon suture was used as an occluder and was inserted via the ECA. The occluder was inserted through a stump of the ECA and the CCA was kept intact. The occluder was advanced into the ICA about 20 mm beyond the carotid bifurcation. Mild resistance indicated that the occluder was properly lodged in the anterior cerebral artery and thus blocked blood flow to the middle cerebral artery. At 1 h after MCAO, reperfusion began with the suture withdrawn. In sham-operated animals, the suture was inserted 5 mm from the incision.

2.2. Oxygen–glucose deprivation, evaluation of apoptosis and cell viability

For oxygen–glucose deprivation (OGD), glucose-free Earl's balanced salt solution (EBSS) medium supplemented with gentamycin (5 mg/l) was purged with N_2/CO_2 (95%/5%) for 20 min, resulting in an oxygen content of 1%. Neurons were then washed three times with this medium and incubated for 1 h in an oxygen-free N_2/CO_2 (95%/5%) atmosphere. Control cultures were incubated in EBSS with 10 mM glucose. Thereafter, the medium was replaced by standard culture medium. For 24 h after onset of OGD, cultured neurons were stained with the fluorescent DNA-binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and quantification of cell death was performed. Stained nuclei were visualized under epifluorescence illumination (340 nm excitation and 510 nm barrier filter) using a 20× objective. Neurons with condensed and fragmented nuclei were considered apoptotic.

2.3. Electrophysiological recording

Electrophysiological recording was performed in the conventional whole-cell patch-clamp recording configuration under voltage-clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige). The resistance between the recording electrode filled with pipette solu-

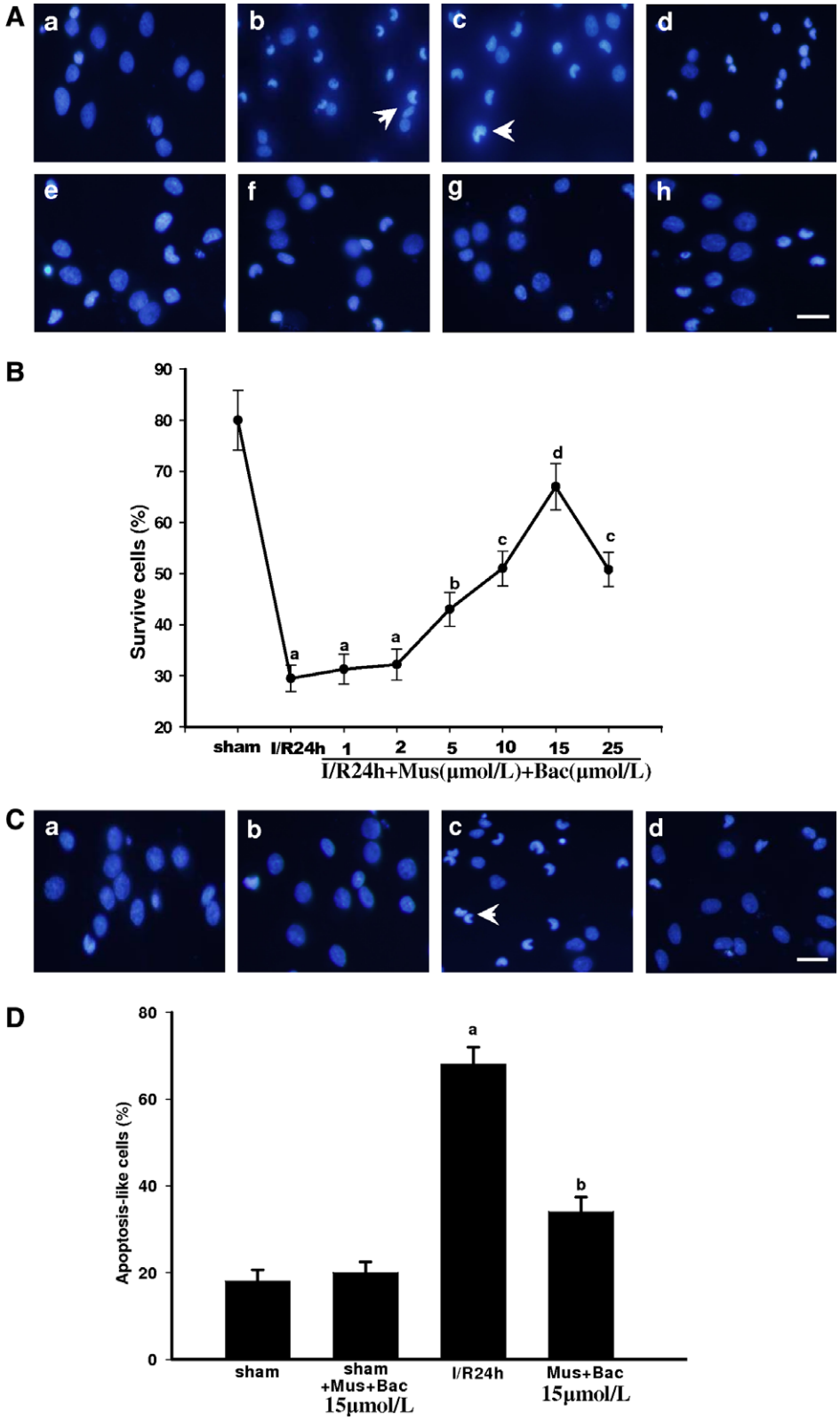

Fig. 2. Muscimol and baclofen attenuates oxygen-glucose deprivation-induced neuronal cell death in hippocampal neurons. (A, B) Staining with DAPI (dark blue) allowed the identification of nuclear. Hippocampal neurons were pretreated with muscimol and baclofen for the indicated concentration 30 min before OGD. Survival cells were indicated as the percentage of every 300 cells. Cell viability in OGD-induced cultures pretreated with muscimol and baclofen was significantly enhanced compared with controls. $^{a}P < 0.05$ versus *I/R* 24 h group, $^{b}P < 0.05$ versus muscimol + baclofen 1–2 μmol/L treatment group, $^{c}P < 0.05$ versus muscimol and baclofen 5 μmol/L or 15 μmol/L treatment group. (C, D) Muscimol and baclofen applied at concentration of 15 μmol/L significantly decreased the OGD-induced apoptosis-like cells. In group b, muscimol and baclofe had no effect on the cell viability without OGD. $^{a}P < 0.05$ versus sham or sham + muscimol + baclofen group, $^{b}P < 0.05$ versus *I/R* 24 h group.

tion and the reference electrode was 3–5 MΩ. Membrane currents were measured using a patch-clamp amplifier AxonPatch 700B (Axon Instruments, Foster City, CA, USA), filtered at 1 kHz, sampled and analyzed using a DigiData 1322A interface and a computer with the pClamp 9.0 system (Axon Instruments). The series resistance was compensated automated. The membrane potential was held at −60 mV throughout the experiment. All experiments were carried out at room temperature (22 – 25 °C).

2.4. Immunohistochemistry

Rats were anesthetized with chloral hydrate and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS). Brains were removed, post-fixed overnight in paraformaldehyde, processed and embedded in paraffin. Coronal brain sections (6-μm thick) were cut on a microtome (Leica RM2155, Nussloch, Germany). Sections were de-paraffinized in xylene and rehydrated in a gradient of ethanol and distilled water. High-temperature antigen retrieval was performed in 1 mM citrate buffer. To block endogenous peroxidase activity, sections were incubated for 2 h in a solution containing 0.01% sodium azide and 0.1% H_2O_2 in PBS. To reduce non-specific staining, sections were incubated for 2 h in a blocking solution containing 1% BSA, 2% normal goat serum, 0.3% Triton X-100 and 5% non-fat dry milk in PBS. The sections were then incubated in the primary antibodies overnight at 4 °C. Alternate sections from each brain were incubated without primary antibody as negative controls. After washes three times in PBS, the sections were incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) made up in 0.1% BSA, 0.3% Triton X-100 and 1% normal goat serum in PBS. Sections were washed and incubated with avidin-conjugated

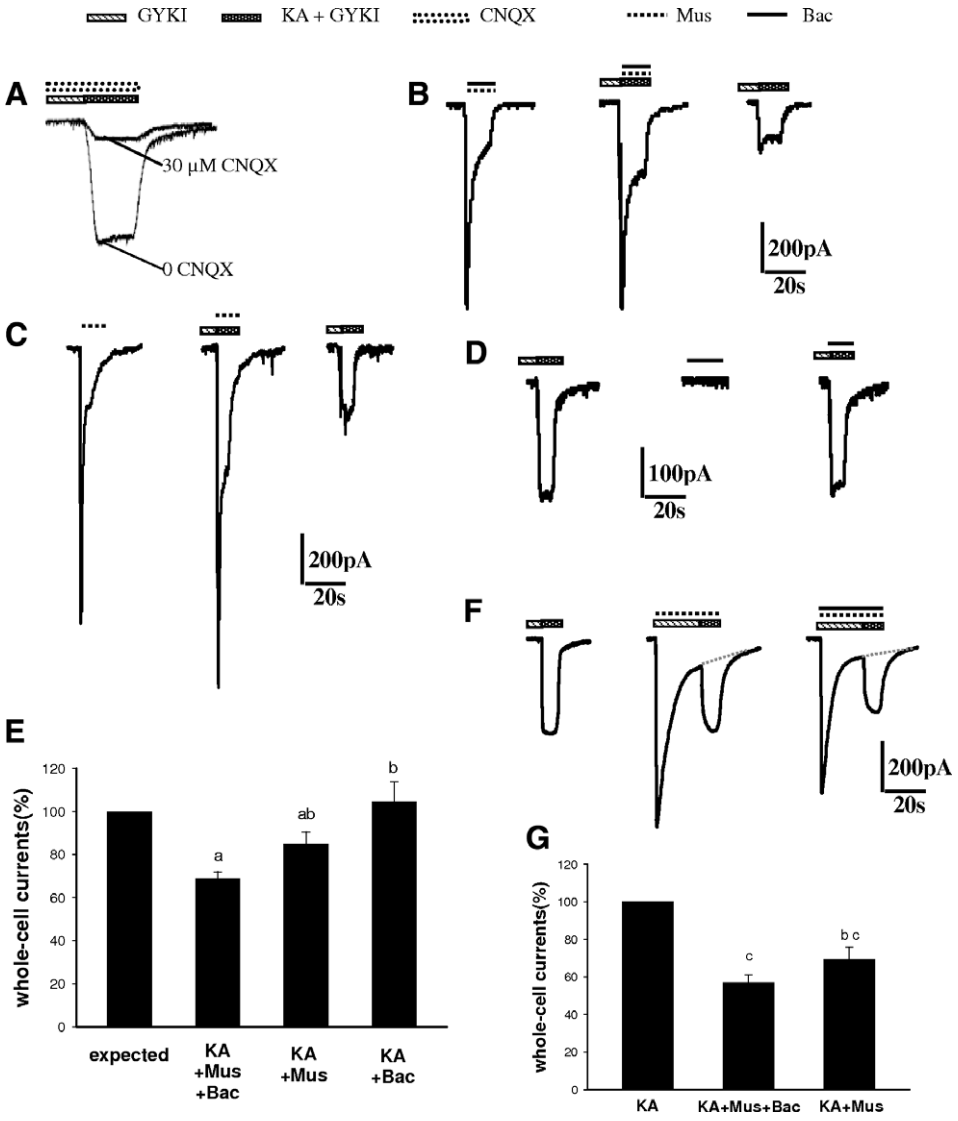

Fig. 3. Cross-inhibition of GABAA and GABAB receptor with KA receptors in cultured rat hippocampal neurons. Co-activation of $GABA_A$ and $GABA_B$ receptor inhibited KA receptor-mediated whole-cell currents in cultured rat hippocampal neurons. (A) KA-induced whole-cell currents were inhibited by non-NMDA GluR inhibitor CNQX. (B) Cross-inhibition of GABA receptors and KA receptors-mediated whole-cell currents. (C, D, E) Co-application of $GABA_A$ receptor activator Mus or $GABA_B$ receptor activator Bac with KA induced whole-cell currents. (F) Effects of single activation of $GABA_A$ receptors and $GABA_B$ receptors on the KA receptor-mediated whole-cell currents. The broken line indicated co-application of Mus and Bac or single application of Mus without KA induced whole-cell currents. (G) Statistic results of GABA receptors' inhibiting effects on KA receptor-mediated whole-cell currents. All data here are expressed as means ± S.D. ($n = 6$). $^aP < 0.05$, compared with the expected group, $^bP < 0.05$ compared with the group of KA + Mus + Bac, $^cP < 0.05$ compared with the group of KA. KA: 100 μM; GYKI 52466: 30 μM; Mus: muscimol 15 μM; Bac: baclofen 15 μM.

horseradish peroxidase (diluted 1:200 in 0.3% Triton X-100) for 1 h at 37 °C, followed by avidin-biotin-peroxidase (ABC, Vector Laboratorie) at room temperature. The sections were examined with a light microscopy.

2.5. Histology

In 4-VO ischemic model, rats were anesthetized with chloral hydrate and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed, post-fixed overnight in paraformaldehyde, processed and embedded in paraffin. Coronal brain sections (6-μm thick) were cut on a microtome. Sections were de-paraffinized in xylene and rehydrated in a gradient of ethanol and distilled water. The sections were stained with Cresyl violet and examined with a light microscope and the neuronal density of the hippocampal CA1 pyramidal cells was expressed as the number of cells per 1-mm length counted under a light microscope (400×).

In MCAO ischemic model, the rats were decapitated 24 h after reperfusion, and their brains were removed and washed with cold saline. The site of the suture tip and reperfusion of the MCA were visually confirmed by the slight dilatation of the vessel without intraluminal thrombosis. The brains were sliced into 2-mm thick coronal sections. The slices were immersed in saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C in the dark for 30 min. Then the stained slices were fixed in 10% formaldehyde. Serial coronal sections were photographed and analyzed by a computer imaging analysis system. The total infarct volume for each brain was calculated by summation of unstained areas of the subsequent slices and multiplying by the thickness. The volume of the damaged hemisphere was calculated respectively. The amount of injury was expressed as a ratio of the total infarct volume to the total volume of the damaged hemisphere.

Other materials and methods used are described in the supplementary data.

3. Results

3.1. Neuroprotective effect of muscimol or/and baclofen on ischemic cerebral injury in vivo

Cresy1 violet staining was used to examine the surviving cells of CA1 pyramidal neurons after 5 days reperfusion. Normal CA1 pyramidal cells showed round and pale stained nuclei. As shown in Fig. 1A and B, transient cerebral ischemia followed by 5 days of reperfusion induced severe cell death (Fig. 1Ac–d, and B). However, administration of muscimol or baclofen 30 min before cerebral ischemia significantly decreased the neuronal degeneration (Fig. 1Ag–j, and B). Moreover, coapplication of muscimol and baclofen significantly increased the survival neurons compared to solely application of muscimol or baclofen (Fig. 1Ai–k, and B). However, vehicle control did not show any protection against the degeneration induced by 5 days of ischemic-reperfusion (Fig. 1Ae–f and B).

Second, we examined the effect of muscimol or/and baclofen on the infarction size of rats subjected to MCAO. Fig. 1C and D present the typical results of the effect of MCA occlusion on infarction size from each of the groups: the MCAO, MCAO-vehicle treatment, and muscimol and baclofen coapplication groups. The white-colored areas represent the infarction regions in these sections. As shown in this figure, coapplication of muscimol and baclofen significantly decreased the amount of injury. These results indicate that administration of muscimol and baclofen have significantly neuroprotective effects against ischemic injury.

3.2. Muscimol and baclofen reduced oxygen-glucose deprivation-induced apoptosis-like cell death in vitro

Hippocampal neurons were pretreated with muscimol and baclofen for the indicated concentration 30 min before OGD, and then cells were stained with DAPI. As shown in Fig. 2A and B, coapplication of muscimol and baclofen significantly enhanced the cell viability against oxygen-glucose deprivation in hippocampal neurons in a dose-dependent manner, with maximum level at 15 μmol/L. Administration of muscimol and baclofen at concentration of 15 μmol/L had no effect on the cell viability without OGD, but significantly decreased the OGD-induced apoptosis-like cells (Fig. 2C and D).

3.3. Cross-inhibition of GABA(A) and GABA(B) receptor with KA receptors in cultured rat hippocampal neurons

Patch-clamp recording was used to detect the KA receptors-mediated whole-cell currents (I_{KA}) and the effects of $GABA_A$ and $GABA_B$ receptors' activators, muscimol (Mus) and baclofen (Bac), on I_{KA}. Muscimol and baclofen induced an intracellular current, and co-application of Mus and Bac with KA evoked the current with an amplitude that was significantly lower than the expected sum of currents evoked by Mus, Bac and KA, respectively (68.7% ± 3.3%). The results suggested that there was a cross-inhibition between $I_{Mus+Bac}$ and I_{KA}(Fig. 3A, B and E). Co-application of Mus with KA also evoked a lower whole-cell currents than the expected sum of Mus and KA (84.8% ± 5.7%), whereas Bac did not induce whole-cell current and had no obvious effect on I_{KA}(104.7% ± 9.1%, Fig. 3C–E). Moreover, GABA receptor activators application was followed with KA application to further investigate relationship between GABA receptor and KA receptor (Fig. 3F and G). Following co-application of Mus and Bac or single application of Mus, I_{KA} became much less than control KA receptor-mediated whole-cell currents (56.9% ± 4.2% and 69.4% ± 6.4%, respectively).

3.4. Muscimol and/or baclofen suppress the increased assembly of the GluR6-PSD-95-MLK3 signaling module induced by ischemic-reperfusion

In our previous study, we found that the interaction of GluR6 and MLK3 with PSD-95 reached its peak level at 6 h reperfusion after 15 min ischemia [7]. Immunoprecipitation and immunoblotting were used to examine the association of GluR6 and MLK3 with PSD-95 after 15 min ischemia followed by 6 h reperfusion in global ischemia model, reciprocal immunoprecipitation experiments were carried out to confirm the results. As shown in Fig. 4A–D, the interaction of GluR6 and MLK3 with PSD-95 increased after 15 min ischemia followed by 6 h reperfusion. Administration of muscimol and/or baclofen 30 min prior to ischemia significantly diminished the increased interaction of GluR6, MLK3 with PSD-95. Meanwhile the protein level of GluR6, PSD-95 and MLK3 were not altered. Note that the effect of coapplication was significant when compared to solely application of either muscimol or baclofen.

3.5. Muscimol and/or baclofen inhibit the activation of MLK3, MKK4/7 and JNK3 during reperfusion after cerebral ischemia

Previous studies indicated that MLK3, an upstream kinase of MKK4/7 and JNK3, could be activated via GluR6 and PSD-95 [7]. As shown in Fig. 5A and B, 6 h reperfusion after 15 min ischemia resulted in remarkable increase in the phosphorylation of MLK3. Pretreatment of muscimol and/or baclofen significantly diminished the increase in the

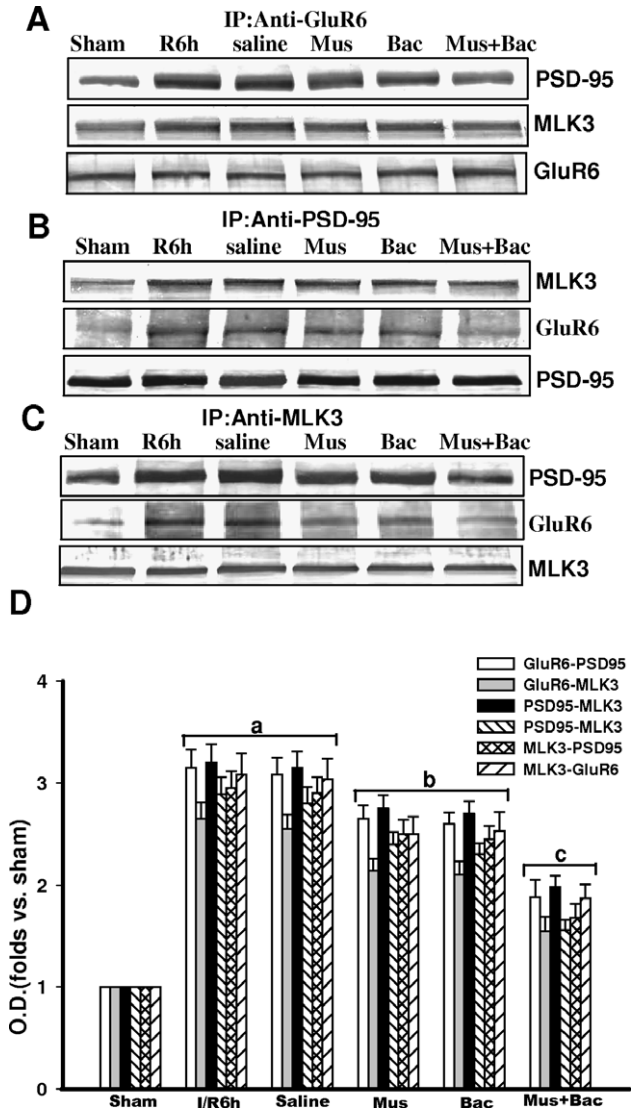

Fig. 4. Effects of pretreatment with muscimol and/or baclofen on the increased interactions of GluR6 and MLK3 with PSD-95 induced by 6 h of reperfusion following cerebral ischemia (*I/R* 6 h) in rat hippocampus. (A, B, C) Co-immunoprecipitation analysis of the interactions among GluR6, PSD-95 and MLK3. Sample proteins were immunoprecipitated (IP) with anti-GluR6, anti-PSD-95 or anti-MLK3 antibody and then blotted (IB) with anti-PSD-95, anti-MLK3 or anti-GluR6 antibody. (D) Quantitative representation of interactions of GluR6 and PSD-95 with MLK3. Values are represented as means ± S.D. for four independent experiments ($n = 4$). $^{a}P < 0.05$ versus sham; $^{b}P < 0.05$ versus saline treatment; $^{c}P < 0.05$ versus muscimol or baclofen treatment group.

phosphorylation of MLK3, while the protein level of MLK3 was not changed. The same dose of control vehicle did not affect the increased phosphorylation of MLK3. Results of western blotting revealed that activation of MKK4/7 was significantly suppressed by application of the muscimol and/or baclofen (Fig. 5C and D). Our previous studies have shown that JNK3 is significantly activated with two peaks at 30 min and 3 days of reperfusion after transient cerebral ischemia [7]. The latter peaks are considered to account for delayed neuronal death because prolonged JNK activation above a threshold might be required for brain cell death. We then investigated the phosphorylation of JNK3 at 3 days of reperfusion. Results revealed that activation of JNK3 was significantly suppressed by application of the muscimol and/or baclofen (Fig. 5E and F).

3.6. *Muscimol and/or Baclofen inhibit the activation of c-Jun and the expression of Fas-L induced by ischemic-reperfusion*

Our previous studies indicated that p-c-Jun could be activated to reach its peak level at 6 h reperfusion after 15 min ischemia [10]. Western blotting was used to examine the phosphorylation of c-Jun. As shown in Fig. 6A and B, muscimol and/or baclofen can significantly prevented the increased c-Jun activation induced by ischemia, without significant effects on the expression of c-Jun, which was confirmed by the result of immunohistochemistry (Fig. 6C).

Further more, the expression of Fas-L and Fas were analyzed by western blotting. As indicated in Fig. 6D and E, the expression of Fas-L was significantly increased 6 h after reperfusion. However, application of muscimol and/or baclofen could diminish the increased expression of Fas-L induced by cerebral ischemic-reperfusion.The same dose of control vehicle did not affect the increase on the expression of Fas-L. The protein level of Fas was not affected. As shown in Fig. 6F, the results of immunohistochemistry also revealed that weak Fas-L immunoreactivity was detected in the cytosol of hippocampal CA1 in the sham group (Fig. 6Fa–b). On the contrary, Fas-L immunoreactivity was significantly increased after 6 h reperfusion (Fig. 6Fc–d) compared with the sham group. No inhibitory effects of control vehicle on Fas-L immunoreactivity were detected (Fig. 6Fe–f). However, Fas-L immunoreactivity after 6 h reperfusion was significantly inhibited by application of muscimol and/or baclofen (Fig. 6Fg–l).

Further results are included in the supplementary data.

4. Discussion

The goal of the present study was to evaluate whether stimulation of GABA receptors has neuroprotective effects and the potential mechanisms underlying these effects. Because now there are conflicting evidence about those activating GABA receptors is directly neuroprotective. Activation of GABA receptors has been said to be protective [11–13] or not [14]. However, our data presented in this study demonstrate that pretreatment either with muscimol (GABA(A) receptor agonist) or baclofen (GABA(B) receptor agonist) has neuroprotective effects either in vivo or in vitro, and the coapplication is more effective than single use. Muscimol and baclofen are two currently used agonists. Both agonists have been tested in experiment ischemia models, and show neuroprotective effects, respectively [15,16]. In the present work, cerebral ischemic model including global cerebral ischemia and focal cerebral ischemia (MCAO) in vivo, and OGD model of primary cultured hippocampal neurons in vitro were adopted to further test the neuroprotective effects of inhibitory GABA signal. We report for the first time that GABA receptors stimulation can diminish the increased association of PSD-95 with GluR6 and MLK3 induced by global ischemia, inhibit the activation of MLK3/JNK3/c-Jun pathway, the Bcl-2 phosphorylation, the expression of Fas-L, the releases of Bax from Bcl-2/Bax dimmers and cytochrome *c* from mitochondria, and the activation of Caspase 3 during reperfusion after cerebral ische-

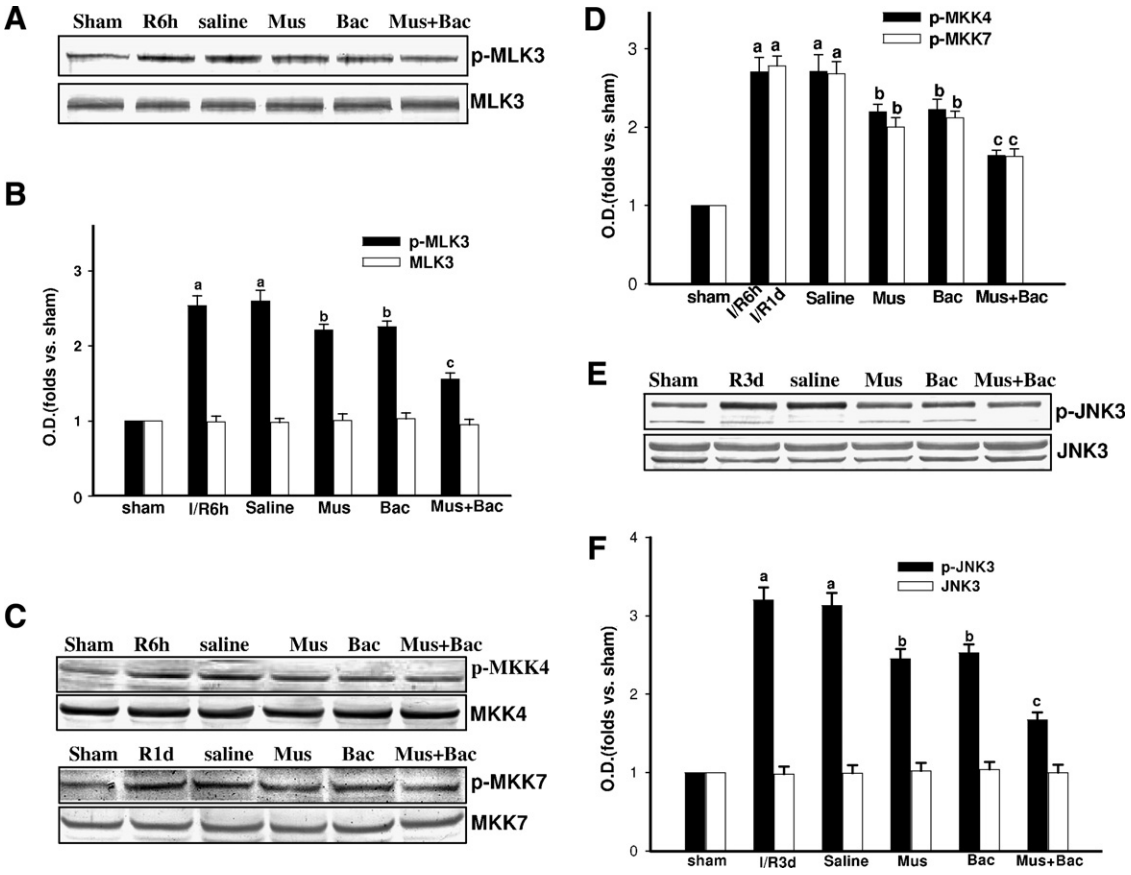

Fig. 5. Effects of muscimol and/or baclofen on the activation of MLK3, MKK4/7 and JNK3 in the hippocampal CA1 region after cerebral ischemia. (A, C, E) Extracts from the CA1 region were subjected to immunoblotting analysis with anti-*p*-MLK3, anti-MLK3, anti-*p*-MKK4/7, anti-MKK4/7, anti-*p*-JNK3 and anti-JNK3 antibodies, respectively. (B, D, F) Data are expressed as means ± S.D. derived from four independent animals ($n = 4$). [a]$P < 0.05$ versus sham group; [b]$P < 0.05$ versus saline treatment group; [c]$P < 0.05$ versus muscimol or baclofen treatment group.

mia. The neuroprotective effects of muscimol and/or baclofen were significant when applied both in vivo and in vitro. Moreover, electrophysiological recording showed that kainate receptor (KAR)-mediated whole-cell currents could be inhibited by co-activation of GABA receptors in cultured rat hippocampal neurons. Taken together, these results suggest that activation of GABA receptors have neuroprotective effects when both agonists were applied solely or together.

The normal functions of brain depend on the dynamic balance of excitation and inhibition. However, cerebral ischemia can both induce the increase of excitation and the decrease of inhibition, which leads to neuronal exitotoxicity. Recently, we found that JNK activation induced by the GluR6-PSD-95-MLK3 module via KAR play a causal role in cerebral ischemia [7,8]. In the present study, we firstly find that elevating the inhibition of GABA receptors can significantly decrease KAR-mediated excitation after ischemic-reperfusion. The major finding is that Muscimol and/or Baclofen can suppress the assembly of the GluR6-PSD-95-MLK3 signaling module during reperfusion after cerebral ischemia. One possible explanation is that the activation of GABA(A) receptor by muscimol can induce the hyperpolarization of postsynaptic nrurons via activating ligand-gated Cl-channels, which decrease the depolarization of the neurons [17], and activation of G-protein-coupled GABA(B) receptor by baclofen can attenuate glutamate release from presynaptic neurons [18]. The inhibition of excitation after GABA receptors activation may thereby attenuate the increased association of PSD-95 with GluR6 and MLK3 induced by ischemic excitotoxicity.

Previous studies have provided a detailed characterization of presynaptic and postsynaptic inhibitory actions of GABA(B) receptors in the hippocampus [19,20]. Presynaptically located GABA(B) receptors modulate neurotransmitter release by depressing Ca^{2+} influx [21], while postsynaptic GABA(B) receptors is primarily mediated by G-protein-coupled inwardly rectifying K^+ channels[22]. The widely distributed GABA(B) protein throughout the hippocampal formation suggest a underlying cross-talk and mutual regulation between GABA(A) and GABA(B) receptors. Indeed, numerous reports illustrate that GABA(A) and GABA(B) receptors can mutually influence each other's ligand binding properties [23,24] and signaling activity [23,25–27]. Recently, Balasubramanian et al. [28] found that GABA(B) receptors and GABA(A) receptor subunits are physically associated in native brain tissue. GABA(B)R1 surface expression can increase the potency of GABA(A) receptors, and GABA(A) receptor γ2S subunit promotes GABA(B)R1 cell surface expression in the absence of GABABR2. These data suggest that agonist activation of GABA receptors can be mutually modulated by the association between these two different classes of GABA receptor. Additionally, Kulinskii and Mikhel'son [29] have found that GABA(B) receptors agonist baclofen and GABA(A) receptors agonists THIP and muscimol are additively contribute to the improvement of brain resistance to global ischemia. As to

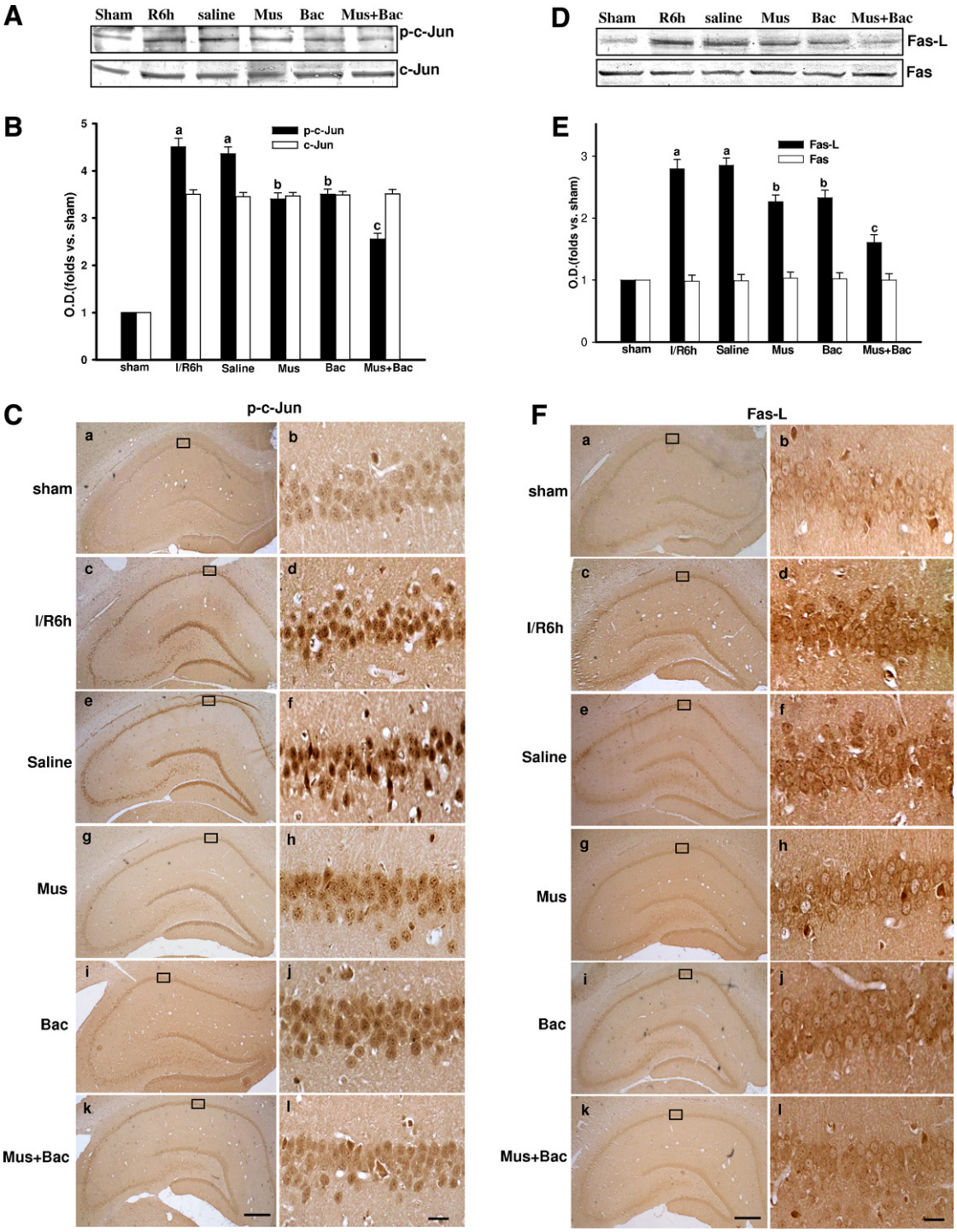

Fig. 6. Effects of muscimol and/or baclofen on the increased c-Jun phosphorylation and protein level of Fas-L in the hippocampal CA1 region after cerebral ischemia. (A, D) Phosphorylation of c-Jun and the protein levels of c-Jun, Fas-L and Fas were examined by immunoblotting analysis with the indicated antibody. (B, E) Bands on Western blots were scanned and the intensities were expressed as folds versus sham treatment. Values are represented as means ± S.D. for four independent experiments ($n = 4$). [a]$P < 0.05$ versus sham group; [b]$P < 0.05$ versus saline treatment group; [c]$P < 0.05$ versus muscimol or baclofen treatment group. (C, F) Immunohistochemistry analysis of the phosphorylation of c-Jun and the expression of Fas-L in coronal sections of hippocampi at 6 h of reperfusion. Representative results on five independent experiments ($n = 5$) were shown from immunohistochemical staining sections of the hippocampi from sham operated rats (a, e), rats subjected to 15 min of ischemia followed by 6 h of reperfusion (c, d), and rats with administration of vehicle (e, f), muscimol (g, h), baclofen (i, j), muscimol and baclofen (k, l) 30 min before ischemia. Boxed areas in left column are shown at higher magnification in right column. Scale bar: $k = 400$ μm, $l = 10$ μm.

our findings in present study that coapplication of GABA(B) receptors and GABA(A) receptors agonists are more effective than solely used, our observations further suggest that these two receptor types may cooperate to produce inhibitory synaptic transmission.

Further discussion is included in the supplementary data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.044.

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